



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Attorney Docket No.: PENN-0857

Inventors: Eberwine and Madison

Serial No.: 10/630,333

Filing Date: July 30, 2003

Examiner: Not yet assigned

Group Art Unit: Not yet assigned

Title: Simplified Use of 5' Ends of RNAs for
Cloning and cDNA Library Construction

"Express Mail" Label No. **EL976563054US**
Date of Deposit - **December 31, 2003**

I hereby certify that this paper is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Commissioner for Patents, Mail Stop Sequence, P.O. Box 1450, Alexandria, VA 22313-1450.

By Kathleen A. Tyrrell
Typed Name: Kathleen A. Tyrrell, Reg. No. 38,350

Commissioner for Patents
Mail Stop Sequence
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

AMENDMENT

In response to the "Notice to Comply" dated **November 6, 2003**, a response to which is due **January 6, 2004**, please amend the specification as follows.

Amendments to the specification begin on page 2.

Remarks begin on page 5.

Please enter the following amendments to the specification:

Please replace the paragraph at page 3, line 32 through page 5, line 4, with the following:

The present invention provides a method of directional cloning which uses the 5'ends of RNAs, for example, to obtain cDNA clones. A detailed schematic of the method of the present invention being used to produce cDNA clones is provided in Figure 2. In this embodiment, oligo-dT or random priming of poly A+ mRNA is used to generate (-) first strand cDNAs. These cDNAs are then homopolymerically tailed with dG or dC using terminal deoxynucleotidyl transferase. After tailing, the heteroduplex is denatured by heat and the mRNA removed by alkaline hydrolysis or RNase digestion to yield single-stranded (-) cDNA. The single-stranded (-) cDNA is then mixed with an first oligonucleotide incorporating a palindromic restriction site in the middle which is flanked on both the 5' and 3' sides with at least two completely degenerate nucleotides. In a preferred embodiment the first oligonucleotide consists of ten bases, including a 6 base palindromic restriction site, such as that for *EcoRI*, flanked by two degenerative nucleotides, as the ten base overall length allows a high degree of specificity of targeting with reasonable annealing temperature. Exemplary sequences are depicted in SEQ ID NO:1 and 2, step 4 of Figure 2a-2b. However, this oligonucleotide can be longer to incorporate other palindromic restriction endonuclease recognition sequences. Examples of restriction endonuclease recognition sequences which can be used include, but are not limited to, *BglIII*, *ClaI*, *EcoRV*, *SacI*, *KpnI*, *SmaI*, *BamHI*, *XbaI*, *SalI*, *AccI*, *AvaI*, *PstI*, *SphI*, *HindIII*, *HincII*, *NsiI*, *NotI*, *SfiI*, *ApaI*, *NcoI*, *StuI*, *NdeI*, *PvuII*, and *XhoI*. After mixing, the oligonucleotide-cDNA mixture is slowly cooled from 50°C to 37°C and the cognate restriction enzyme is added. The resulting annealed, short double-stranded DNA segments correspond to the positions of

these restriction sites on the (-) cDNA. Cleavage by the cognate restriction enzyme yields single-stranded cDNAs bound on their 5' end by the "sticky end" left by the restriction enzyme used and on their 3' end by a poly-dG or -dC tract. Thus, the method of the present invention allows specific site-directed cleavage of the single-stranded (-) cDNA thereby eliminating the need for second strand synthesis of the entire (+) cDNA to provide the double-stranded restriction site as in prior art methods. Accordingly, the present invention is much simpler and requires less time than the prior art methods. Further, considerably smaller amounts of oligonucleotide triphosphate reagents are required.

Please replace the paragraph at page 5, line 5 through 26 with the following:

A second oligonucleotide, as exemplified by SEQ ID NO:3, Figure 2b, step 6, comprising nucleotides complementary to the 3' end of the cDNAs and containing the same restriction site as in the first oligonucleotide is then annealed to the 3' poly-dG or -dC tailed single-stranded (-) cDNA by a similar cycle of heating and slow cooling as described above. Since this single-stranded (-) cDNA contains the cognate "sticky end" at its 5' terminus, the 5' end can loop back and also anneal to the second oligonucleotide at the restriction site. The resulting primed and gapped single-stranded (-) cDNA (exemplified in SEQ ID NO:4, Figure 2c, between steps 8 and 9) is stabilized and rendered replication-competent for second strand synthesis of (+)cDNA by the addition of a DNA polymerase and DNA ligase. Since the cDNA region to be replicated is shorter than the original full-length sequence, the likelihood of it being completely and accurately replicated is increases over standard methods requiring the traverse of a longer region of (-) cDNA. The resulting double-stranded closed-circular cDNA is readily separated from linear single-stranded fragments and

trinucleotides by spin column chromatography or agarose gel electrophoresis.